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Appl. Environ. Microbiol. **2013, 79(20):6414. DOI: 10.1128/AEM.01954-13.**
Published Ahead of Print 9 August 2013.

AEM
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Functional and Expression Analysis of the Metal-Inducible *dmeRF* System from *Rhizobium leguminosarum* bv. viciae

L. Rubio-Sanz,[a] R. I. Prieto,[a] J. Imperial,[a,b] J. M. Palacios,[a] B. Brito[a]

Centro de Biotecnología y Genómica de Plantas (CBGP) and Departamento de Biotecnología, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, Madrid, Spain[a]; Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain[b]

A gene encoding a homolog to the cation diffusion facilitator protein DmeF from *Cupriavidus metallidurans* has been identified in the genome of *Rhizobium leguminosarum* UPM791. The *R. leguminosarum dmeF* gene is located downstream of an open reading frame (designated *dmeR*) encoding a protein homologous to the nickel- and cobalt-responsive transcriptional regulator RcnR from *Escherichia coli*. Analysis of gene expression showed that the *R. leguminosarum dmeRF* genes are organized as a transcriptional unit whose expression is strongly induced by nickel and cobalt ions, likely by alleviating the repressor activity of DmeR on *dmeRF* transcription. An *R. leguminosarum dmeRF* mutant strain displayed increased sensitivity to Co(II) and Ni(II), whereas no alterations of its resistance to Cd(II), Cu(II), or Zn(II) were observed. A decrease of symbiotic performance was observed when pea plants inoculated with an *R. leguminosarum dmeRF* deletion mutant strain were grown in the presence of high concentrations of nickel and cobalt. The same mutant induced significantly lower activity levels of NiFe hydrogenase in microaerobic cultures. These results indicate that the *R. leguminosarum* DmeRF system is a metal-responsive efflux mechanism acting as a key element for metal homeostasis in *R. leguminosarum* under free-living and symbiotic conditions. The presence of similar *dmeRF* gene clusters in other *Rhizobiaceae* suggests that the *dmeRF* system is a conserved mechanism for metal tolerance in legume endosymbiotic bacteria.

Nickel and cobalt are essential microelements for microbial nutrition that participate in a variety of cellular processes. In particular, nickel participates as a cofactor in at least nine enzymes, including urease and hydrogenase (1), whereas cobalt is required for activity of corrinoid-containing enzymes such as isomerases and methyl transferases (2). These two elements are usually present at low concentrations in soils, and bacteria have developed high-affinity metal uptake systems for the cations (3). In contrast, moderate concentrations of the same elements can become toxic by displacing other metals from the active site of metalloenzymes, by catalyzing the production of free radicals, or by interfering with the assembly of FeS clusters (4, 5). Nickel homeostasis requires the balance of import and export pathways to control metal concentration inside the bacterial cell (6). Active transport efflux pumps represent the largest category of metal resistance systems (7). Most studies on bacterial metal resistance have been carried out with the heavy metal-resistant organism *C. metallidurans* CH34 (8). In this organism, three main groups of efflux systems have been characterized: RND (resistance, nodulation, and cell division) proteins, cation diffusion facilitators (CDFs), and P-type ATPases (9). Bacteria utilize primarily the first two groups for dealing with Ni(II) and Co(II) (2).

Members of the RND group are membrane proteins that participate in trimeric complexes along with outer membrane factors and bridging periplasmic proteins (10). Such complexes are able to export toxic substances, including heavy metals, acting as a kind of peristaltic pump driven by proton motive force to pump out metals from the periplasm across the outer membrane (11).

CDF proteins use a Me^{2+}/H^{+} proton-antiport mechanism to drive the translocation of heavy metals across membranes (12). CDF substrates are divalent cations such as Zn(II), Mn(II), Cd(II), Fe(II), Zn(II), and Co(II). As a general rule, CDF proteins contain six putative transmembrane domains (TMD) with a C terminus protruding into the cytoplasm and carrying metal binding sites (12). Many CDF transporters also contain a histidine-rich domain to allow more efficient metal binding. At least some of the members of the family function as homo-oligomeric complexes. CDFs are present in organisms from the three kingdoms of life (13). The model example of bacterial CDF is *Escherichia coli* YiiP, a homodimeric protein involved in the efflux of Fe, Zn, and Cd (14). In the metal-resistant bacterium *Cupriavidus metallidurans*, a CDF protein (DmeF, for Divalent metal efflux) is essential for cobalt export to the periplasm (15).

A key aspect of metal homeostasis is the regulation of expression of transporter proteins. Bacteria contain metalloregulatory proteins to fine-tune the expression of genes involved in uptake and efflux of metals (3). Two *E. coli* proteins, NikR and RcnR, are involved in metal sensing and regulation of gene expression in response to nickel or cobalt ions. NikR is a member of the bacteriophage P22 Arc repressor superfamily. This protein controls the expression of Ni uptake genes (*E. coli nikABCDE*) and other nickel-related genes such as urease genes in *Helicobacter pylori* (16). RcnR is a metal-responsive repressor that constitutes, along with the copper regulator CsoR, the most recent addition to the list of major groups of metalloregulators (17, 18). *E. coli* RcnR represses the expression of the Ni- and Co-specific efflux system RcnAB by binding to a specific sequence with G/C tracts flanked by AT-rich inverted repeats in the operator region (19). This repression is

Received 15 June 2013 **Accepted** 6 August 2013
Published ahead of print 9 August 2013
Address correspondence to J. M. Palacios, jose.palacios@upm.es.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01954-13.

doi:10.1128/AEM.01954-13

released upon binding of Ni or Co ions to a histidine-rich motif (H-C-H-H) critical for RcnR function (20).

The symbiotic interaction between *Rhizobium* and legume plants is a key component of sustainable agricultural systems, due to the ability of these endosymbiotic bacteria to fix atmospheric nitrogen into ammonia provided to the plant (21). In this symbiosis, the bacteria infect the legume roots and induce the formation of nodules in which bacteria proliferate and fix nitrogen. A plant-derived peribacteroid membrane surrounds bacteroids, the symbiotic form of the bacteria, thus controlling nutrient exchange between both symbionts (22). Although the *Rhizobium*-legume symbiosis has been proposed as a tool for bioremediation of heavy metal-polluted soils (23, 24), the information on determinants involved in metal resistance in *Rhizobiaceae* is scarce, restricted to a few reports on the levels of metal tolerance by members of this relevant group of endosymbiotic bacteria (25, 26). However, this bacterial group might be a relevant reservoir of genetic determinants mediating survival under high-metal conditions, as deduced from the large number of metal resistance genes identified in the genome of a *Mesorhizobium amorphae* isolate obtained from a Zn/Pb mine tailing (27). In this paper, we describe the functional characterization and expression analysis of an Ni(II)- and Co(II)-inducible system (DmeRF) involved in resistance to these metals in both free-living and symbiotic states of *Rhizobium leguminosarum* bv. viciae.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *R. leguminosarum* bv. viciae strains used in this work were routinely grown at 28°C in tryptone-yeast extract (TY) (28), *Rhizobium* minimal (Rmin) (29), or yeast-mannitol (30) media. Strain SPF25 is derived from UPM791 by replacement of the native NifA-dependent *hupSL* promoter by the FnrN-dependent *fixN* promoter to allow expression of hydrogenase in free-living cells (31). *E. coli* strains DH5α (Bethesda Research Laboratories, United Kingdom) and S17.1 (32) were used for cloning and conjugation purposes, respectively. Antibiotics were added at the following concentrations (in $\mu g \cdot ml^{-1}$): tetracycline, 5; kanamycin, 50; and spectinomycin, 50.

MICs for nickel, cobalt, zinc, and copper were estimated by the ability of the *Rhizobium* strains to grow on TY plates containing increasing concentrations of $NiCl_2$ (0.1, 0.2, 0.3, 0.5, 1.0, 1.5, 2.0, and 2.5 mM), $CoCl_2$ (0.1, 0.2, 0.3, 0.4, 0.5, 0.75, and 1.0 mM), $ZnSO_4$ (0.5, 1.0, 1.5, and 2.0 mM), or $CuSO_4$ (0.5, 1.0, 1.5, and 2.0 mM). For disk diffusion susceptibility tests (33), bacterial cultures grown to the exponential phase were mixed with warm TY agar medium and poured into petri plates. Disks soaked with different amounts of $NiCl_2$ (100, 200, or 500 mM), $CoCl_2$ (20, 50, or 100 mM), $CuSO_4$ (100, 200, or 500 mM), $ZnSO_4$ (100, 200, or 500 mM), and $MnCl_2$ (100, 200, or 500 μM) were placed on the surface. The zone of inhibition was measured after 48 h of incubation at 28°C.

DNA manipulation techniques and plasmid constructions. Plasmid DNA preparations, restriction enzyme digestions, and DNA transformations into *E. coli* were carried out by standard protocols (34). For analysis of promoter expression, transcriptional gene fusions were generated with the promoterless *lacZ* gene present in plasmid pMP220 (35). DNA fragments containing the 5′ end of *dmeR* and *dmeF* genes along with upstream regions were amplified using primer pairs dmeR1_F/dmeR1_R (5′-AGAGCGGCACGAGAATGG-3′/5′-GGACGGAGGCGAGCAGTT-3′) and dmeR2_F/dmeRF_R(5′-TTGAAGGGGCAGATGGAG-3′/5′-GGCACGGGATTGGAAAGG-3′), respectively, cloned in plasmid pCR2.1-TOPO, and subcloned in pMP220 as KpnI/XbaI fragments, thus generating plasmids pDL13 (*dmeR′-lacZ*) and pDL43 (*dmeF′-lacZ*). An additional fusion plasmid containing the region upstream *dmeR*, the whole *dmeR* gene, and the 5′ end of *dmeF* was constructed by a similar strategy using primers dmeR1_F and dmeRF_R (fusion plasmid pDL10, *dmeRF′-lacZ*). These plasmids were introduced into *R. leguminosarum* by mating, and transconjugants were selected in *Rhizobium* minimal medium supplemented with tetracycline.

Generation of the *dmeRF*-deficient mutant strain D15 was carried out by a marker exchange approach. Two DNA regions of ca. 1 kb corresponding to the *dmeR* upstream and *dmeF* downstream regions were amplified by PCR using primer pairs dmeR1_F/dmeR1_R and dmeF_F (5′-TTGTTGCCGTCCTTACCT-3′)/dmeF2_R (5′-CCGCTCCTTGCCTGTCGT-3′), respectively. Both regions were cloned in plasmid pK18*mobsacB* with an intervening DNA fragment containing a spectinomycin resistance cassette. This construction was introduced into *R. leguminosarum* SPF25 by conjugation, and single and double recombination events were selected in *Rhizobium* minimal medium as described previously (36). The *dmeRF* deletion was confirmed through Southern blot analysis of EcoRI-digested genomic DNA from the mutants, using a digoxigenin (DIG)-labeled DNA fragment containing *dmeRF* genes as a probe. Hybridizing bands were visualized using a chemiluminescent DIG detection substrate as described by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

qRT-PCR analysis. For RNA preparation, *R. leguminosarum* SPF25 cells were grown in standard TY medium or in TY medium supplemented with metals (200 μM $NiCl_2$ or 10 μM $CoCl_2$) and incubated for 24 h at 28°C. Cells were harvested from 5 ml of culture by centrifugation and resuspended in 500 μl of Tris-EDTA (TE) buffer, and RNA was stabilized with RNA Protect Bacteria reagent (Qiagen, Hilden, Germany) and purified with an RNeasy minikit (Qiagen). Contaminating DNA was removed with Turbo DNA-free (Ambion, Life Technologies Ltd., Paisley, United Kingdom). cDNAs were obtained using SuperScript III reverse transcriptase (Invitrogen Life Technologies Ltd., Paisley, United Kingdom) according to the manufacturer's instructions. Quantitative reverse transcription-PCR (qRT-PCR) was carried out with a Power SYBR green master mix (Applied Biosystems Life Technologies Ltd., Paisley, United Kingdom) in a final volume of 25 μl.

For transcript analysis of *dmeF*, *orf03473*, and *orf03476*, cDNA was used as the template for qRT-PCR using primer pairs qdmeF(5′-AGGACGCTGCCGATACAA-3′)/qdmeR(5′-TCCTGCCGTTGTTAACGC-3′), orf03476F(5′-GACACGCTCGGCAATCTGAC-3′)/orf03476R(5′-GCACGGTCGTCTCGCTGATA-3′), and orf03473F(5′-CCATTCTCGTGCCGCTCTAC-3′)/orf03473R(5′-GGGTGAAATCCAGCTGTTCG-3′), respectively. Primers rpoD_F (5′-GATGAAGTCGATCGGCAATCTG-3′) and rpoD_R (5′-GCTTCGACCATTTCCTTCTTGG-3′) were used to estimate expression of *rpoD* as an internal reference. The qRT-PCR program consisted of 10 min of incubation at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C and a final cycle of 15 s at 95°C, 60 s at 60°C, 15 s at 95°C, and 15 s at 60°C. Determinations were carried out with RNA extracted from three independent biological samples, with the threshold cycle (C_T) determined in triplicate for each biological replicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta CT}$ method (37). To control DNA contamination, PCRs were performed on RNA preparations prior to reverse transcription using the same primer pairs.

Plant tests and enzymatic activity. Pea (*Pisum sativum* L. cv. Frisson) and lentil (*Lens culinaris* L. cv. Magda) seeds were surface sterilized and planted in Leonard jar-type assemblies under bacteriologically controlled conditions (38). Plants were grown in a greenhouse with 16-h/8-h light-dark cycle and 25/23°C day-night temperature. When required, the nitrogen-free plant nutrient solution was supplemented with 85 μM $NiCl_2$ or 42.5 μM $CoCl_2$ 10 days after seedling inoculation (39).

Plant shoot dry weight was determined after drying at 60°C for 24 h. Nitrogen content of the shoot was determined by a Kjeldhal method (40) with 0.5 g of ground plant material per sample.

β-Galactosidase activities in *R. leguminosarum* free-living cultures and in bacteroid suspensions obtained from 21-day-old plants were determined as described by Miller (41). For this purpose, free-living cells were grown in TY liquid medium overnight, diluted in fresh TY medium supplemented with increasing concentrations of $NiCl_2$ or $CoCl_2$, and incubated for 24 h at 28°C. The assays were conducted using *ortho*-nitrophe-

TABLE 1 Effect of deletion of *dmeRF* genes on nickel and cobalt resistance in *R. leguminosarum* bv. viciae SPF25

Strain	Genotype	Medium	MIC (mM)[a]				Inhibition zone (mm)[b]			
			$NiCl_2$	$CoCl_2$	$CuSO_4$	$ZnSO_4$	$NiCl_2$	$CoCl_2$	$CuSO_4$	$ZnSO_4$
SPF25	Wild type	TY	1	0.75	2	2	23	21	17	20
D15	Δ*dmeRF*	TY	0.75	0.3	2	2	27	30	18	20
SPF25	Wild type	Rmin	0.1	0.2	ND	ND	ND	ND	ND	ND
D15	Δ*dmeRF*	Rmin	0.05	0.05	ND	ND	ND	ND	ND	ND

[a] Values represent the results of three separate experiments.
[b] Values are the averages of three replicates. Standard errors were below 5%. ND, not determined.

nyl-β-D-galactopyranoside (ONPG) as the substrate. Values for β-galactosidase activity were calculated as Miller units. The protein contents of bacteroid suspensions and free-living cultures were measured by the bicinchoninic acid method (42) with the modifications described by Brito et al. (43).

RESULTS

Identification of *dmeRF* genes in *R. leguminosarum* bv. viciae. The cation diffusion facilitator (CDF) encoded by *dmeF* has a key role in resistance to nickel and cobalt in the metal-resistant strain *C. metallidurans* CH34 (15). In order to identify potential genetic systems involved in nickel and cobalt resistance in *R. leguminosarum* bv. viciae, the genome of this bacterium (JGI-128C53, Gi08894) was analyzed for homologs to this gene. An open reading frame (ORF; *orf03475*) encoding a protein with 39% amino acid identity to *dmeF* was identified. This ORF encodes a 343-amino-acid protein with a predicted structure including six transmembrane (TM) domains (see Fig. S1 in the supplemental material). In this protein we also identified the two motifs characteristic of CDF proteins identified by Montanini et al. (13) and a histidine-rich stretch located between TM4 and TM5, so we designated the *R. leguminosarum* gene *dmeF*. A phylogenetic tree (see Fig. S2 in the supplemental material) built with proteobacterial CDF protein sequences grouped *R. leguminosarum dmeF* within the Zn-CDF group previously defined (13).

Upstream of *dmeF*, the *R. leguminosarum* UPM791 genome presents an ORF encoding a 90-amino-acid protein showing high similarity (39% identical residues) to *E. coli* RcnR, one of the founding members of the RcnR/CsoR structural class of metal-responsive transcriptional regulators (17, 44). Alignment of the two proteins revealed that the *R. leguminosarum* gene product contained a conserved cysteine residue (Cys-35) and three out of the four conserved histidine residues (His-3, His-60, and His-64) involved in response to Ni(II) and Co(II) (20, 45); furthermore, structural prediction based on I-TASSER software (46) indicated the presence of three alpha helices similar to those present in CsoR/RcnR (see Fig. S1). Based on the regulatory role of this protein in *R. leguminosarum* (see below), the corresponding gene was designated *dmeR*.

Functional analysis of *dmeRF* in *R. leguminosarum* free-living and symbiotic cells. In order to carry out the functional analysis of *dmeRF* genes, a Δ*dmeRF*::Spc^r mutant of *R. leguminosarum* SPF25 was constructed by replacement of these genes with a spectinomycin resistance cassette, thus resulting in strain D15.

We first analyzed the effect of the elimination of *dmeRF* genes on the levels of nickel and cobalt resistance. As shown in Table 1, inactivation of *dmeRF* genes led to decreased levels of tolerance to nickel and cobalt both in rich and minimal media (TY and Rmin). Similarly, assays with disks soaked with Ni(II) or Co(II) solutions revealed significant increases in the size of inhibition halos for these two metal ions, but not in the case of Zn(II) or Cu(II). All these data indicate that the *dmeRF* system is involved in resistance to nickel and cobalt in *R. leguminosarum*.

We tested the potential relevance of *dmeRF* genes to the symbiosis with host legumes grown under low and high levels of metals. To this aim, pea plants inoculated with wild-type *R. leguminosarum* SPF25 or with its *dmeRF*-deficient derivative D15 were grown for 3 weeks under greenhouse conditions either with a standard nutrient solution or with the same solution supplemented with nickel or cobalt. The data obtained (Table 2) indicate that the mutation had no significant effects on symbiotic performance

TABLE 2 Effect of deletion of *dmeRF* genes on symbiotic performance of *R. leguminosaum* bv. viciae with pea and lentil as plant hosts

Strain[a]	Concn of metal added (μM)		Pea		Lentil	
	Ni(II)	Co(II)	Shoot DW[b]	N fixed[c]	Shoot DW[b]	N fixed[c]
SPF25	0	0	269.6 ± 25.3	10.8 ± 1.5	196.2 ± 23.3	5.2 ± 0.7
D15	0	0	249.9 ± 32.0	9.9 ± 1.3	177.9 ± 38.6	4.8 ± 1.2
Control[d]	0	0	155.7 ± 24.8*	1.8 ± 0.2*	120.1 ± 21.1*	1.4 ± 0.1*
SPF25	85	0	220.1 ± 46.9	8.4 ± 1.6	190.7 ± 54.4	4.9 ± 1.9
D15	85	0	141.7 ± 47.6	5.4 ± 2.0	195.2 ± 40.2	5.2 ± 1.5
SPF25	0	42.5	293.8 ± 45.6	11.3 ± 1.7	182.9 ± 38.0	4.7 ± 1.0
D15	0	42.5	218.3 ± 40.8*	8.8 ± 1.9*	174.6 ± 12.3	4.6 ± 0.6

[a] *R. leguminosarum* strains used were SPF25 (wild type) and D15 (*dmeRF* deletion derivative).
[b] Values of shoot dry weight (DW) (mg · plant^{-1}) correspond to the averages of at least three replicates ± standard errors. *, statistically significant difference (analysis of variance, $P < 0.05$) from the wild type grown under the same conditions.
[c] Values (mg of N · plant^{-1}) correspond to the averages of at least three replicates ± standard errors.*, statistically significant difference (analysis of variance, $P < 0.05$) from the wild type grown under the same conditions.
[d] As a control, noninoculated plants were used to verify the absence of cross-contamination.

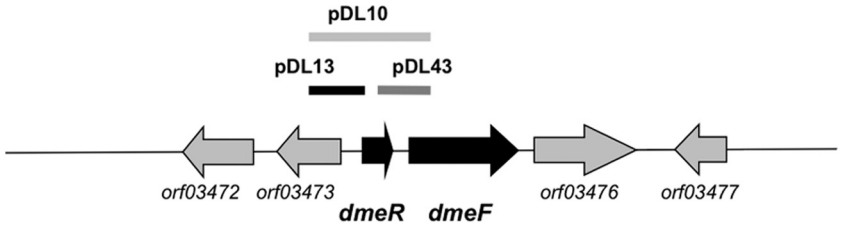

FIG 1 Genetic map of *dmeRF* genes in the *R. leguminosarum* bv. viciae UPM791 genome. Thick lines above the genetic map indicate the DNA regions cloned in pMP220 in the indicated fusion constructs. ORF designation corresponds to those used in the Joint Genome Institute database (Gi08894).

when plants were grown with standard nutrient solutions. In the case of plants supplemented with cobalt, however, deletion of the *dmeRF* system resulted in a statistically significant decrease (26%) of the average dry-weight values in the mutant compared to the wild type. Similar reductions in the average values were observed in the case of nickel, although in this case differences were not statistically significant, likely due to the high heterogeneity of the plants. These results indicate that the *dmeRF* system has a relevant role in the symbiotic performance of the pea-*R. leguminosarum* association when pea plants were grown under high-metal conditions. We also tested the effect of the *dmeRF* system on the symbiotic performance of lentil plants. This alternative host is known to provide lower levels of nickel to the bacteroids, thus resulting in reduced levels of hydrogenase activity (39). In the case of lentils, symbiotic performance was not affected by the deletion of the *dmeRF* system under either low- or high-metal conditions (Table 2). We interpret this result as a consequence of the lower level of metals available to the bacteroids in this plant species, thus avoiding noxious metal buildup in the absence of the efflux system.

Expression of *dmeRF* genes is induced by nickel and cobalt in *R. leguminosarum*. Since metal efflux systems from *E. coli* and other bacteria are regulated by the presence of the corresponding metal cation (2), we decided to study the effect of the addition of metal ions on the expression of *dmeRF* genes. Expression analysis of *dmeRF* genes was performed first by using fusions to the *lacZ* reporter gene. The DNA region containing the *dmeR* upstream region along with the *dmeR* gene and the 5′ end of *dmeF* was cloned into the pMP220 vector to obtain the *dmeRF′-lacZ* fusion plasmid pDL10 (Fig. 1). Also, the *dmeR* and *dmeF* upstream regions were cloned independently in vector pMP220 to generate transcriptional fusion plasmids pDL13 (*dmeR′-lacZ*) and pDL43 (*dmeF′-lacZ*). These plasmids were introduced into *R. leguminosarum* strains SPF25 and D15, and the reporter activity was determined in cell cultures grown in media supplemented with increasing nickel and cobalt concentrations.

Expression of the *dmeRF* genes was analyzed first in free-living cells from *R. leguminosarum* strain SPF25 (Fig. 2). In this background, the *dmeRF′-lacZ* fusion pDL10 was associated with basal

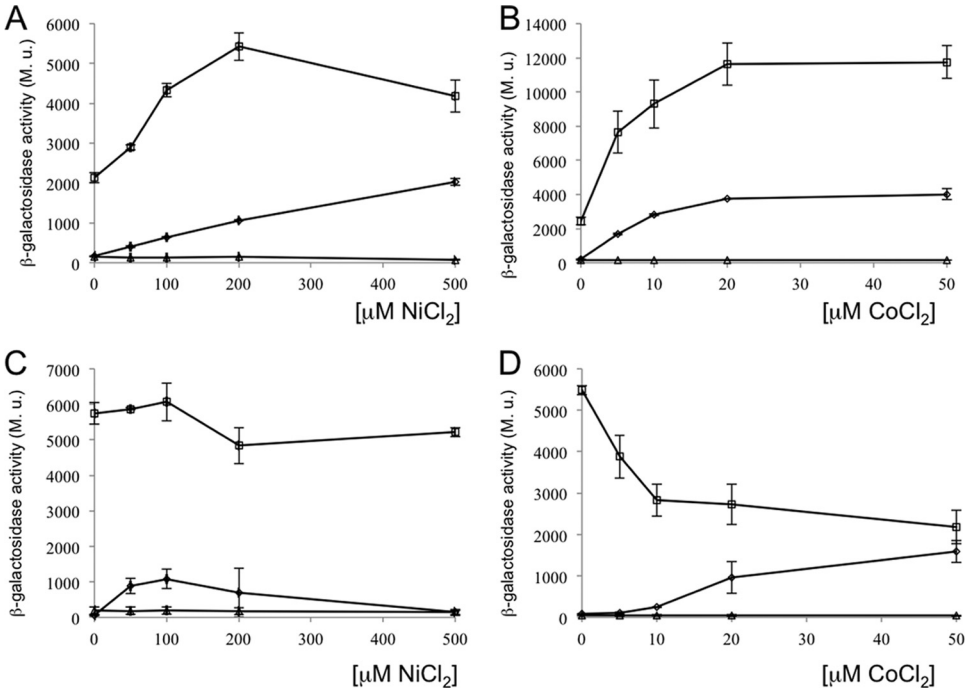

FIG 2 Expression analysis of *R. leguminosarum dmeRF* genes as a function of nickel or cobalt concentration in the culture medium. *R. leguminosarum* wild-type SPF25 (A and B) and *dmeRF* mutant D15 (C and D) containing reporter fusion plasmids pDL10 (*dmeRF′-lacZ*) (diamonds), pDL13 (*dmeR′-lacZ*) (squares), or pDL43 (*dmeF′-lacZ*) (triangles) were grown in TY medium supplemented with the indicated amounts of $NiCl_2$ (A and C) and $CoCl_2$ (B and D). β-Galactosidase activities are expressed in Miller units. Values are averages of three independent experiments. Error bars indicate standard errors.

levels of β-galactosidase activity (below 50 Miller units [MU]) in media with no added metals (Fig. 2A). Interestingly, reporter activity mediated by this fusion gradually increased to more than 2,000 MU when the level of nickel added was increased until 500 μM $NiCl_2$, close to the level of toxicity for this metal. In the case of cobalt, metal-dependent induction of reporter activity was even higher and reached a maximum (ca. 4,000 MU) at a cobalt concentration of 20 μM (Fig. 2B). No significant induction of reporter activity over basal levels was observed when other metals [Zn(II), Cu(II), Mn(II), or Cd(II)] were added at concentrations up to 100 μM (data not shown). When similar experiments were carried out with plasmid pDL43 (*dmeF′-lacZ*), only basal levels of reporter activity were observed, irrespective of the Ni(II) or Co(II) levels present in the medium. We conclude from these results that expression of *dmeRF* is induced in response to the presence of nickel and cobalt metal ions from a promoter region located upstream of *dmeR* gene. The existence of a transcriptional unit including both *dmeR* and *dmeF* was confirmed by RT-PCR experiments carried out with cDNA from *R. leguminosarum* cultures in media either supplemented or not supplemented with Ni(II) (see Fig. S3 in the supplemental material).

The reporter expression associated with plasmid pDL13 (*dmeR′-lacZ*) was also analyzed. In this case, significantly higher levels of β-galactosidase activity were detected under all conditions tested, even in the absence of added metals in the medium. For this fusion, the addition of increasing concentrations of nickel or cobalt resulted in higher values of reporter activity, with maxima at ca. 200 μM Ni(II) and 20 μM Co(II) (5,000 and 10,000 MU, respectively), likely due to repressor titration by the higher number of copies of the *dmeRF* promoter region.

When the reporter fusions were introduced into the *dmeRF* mutant D15, activity profiles associated with fusion plasmid pDL10, containing a whole copy of the *dmeR* gene, again showed a Ni(II)- and Co(II)-dependent regulation, whereas those associated with pDL13 were quite different (Fig. 2C and D). In this genetic background, the *dmeR′-lacZ* fusion induced very high β-galactosidase activities even in the absence of added nickel or cobalt in the medium. These data strongly suggest that DmeR acts as a repressor of *dmeRF* expression and that the DmeR protein synthesized from the *dmeR* gene cloned in plasmid pDL10 restores the metal-dependent control of expression of the *dmeRF* promoter. Titration of DmeR would also explain the high values of reporter activity associated with fusion pDL13 in the wild-type strain.

The metal-responsive induction of *dmeRF* genes deduced from the *lacZ* fusion assays was confirmed by qRT-PCR experiments. In this analysis (Fig. 3), we found that the presence of Ni(II) (200 μM) or Co(II) (10 μM) in the medium induced 6-fold and 8-fold increases, respectively, in the level of transcription of the *dmeF* gene, whereas the level of expression of flanking genes *orf03473* and *orf03476* was not modified by the addition of the cations. The lack of metal-induced expression of *orf03476*, along with the basal levels of expression associated with the *dmeF-lacZ* fusion, and the results of RT-PCR experiments (see Fig. S3 in the supplemental material) indicate that the *dmeRF* genes constitute an operon whose expression is induced in response to the presence of Ni(II) and Co(II) ions. Our data also indicate that the expression of *dmeRF* is negatively controlled by the product of *dmeR* and that this repression is likely alleviated by the presence of these cations.

Expression of *dmeRF* genes was also studied in pea bacteroids from SPF25 and *dmeRF*-deficient mutant D15 derivatives carrying each of the reporter gene fusions (Table 3). In this analysis, the wild-type strain *R. leguminosarum* SPF25 exhibited low levels of reporter activity in the case of fusion plasmid pDL10 (*dmeRF-lacZ*), with values similar to those associated with plasmid pDL43 (30 to 40 Miller units), considered the basal level. In the same experiments, the presence of *dmeR-lacZ* fusion plasmid pDL13 was associated with slightly higher reporter activities, irrespective of whether Ni(II) or Co(II) was added to the plants. Our interpretation of these results is that the product of the genomic copy of *dmeR* is able to repress the *dmeRF* promoter under symbiotic conditions. Analysis of the same fusions in bacteroids of the *dmeRF* mutant revealed that expression of these genes was significantly enhanced by cobalt. Under these conditions, the *dmeR-lacZ* fusion was associated with higher levels of unregulated expression in this genetic background (ca. 350 MU). Again, the high reporter activities associated with this fusion are likely the result of the absence of an active copy of *dmeR* in this genetic background.

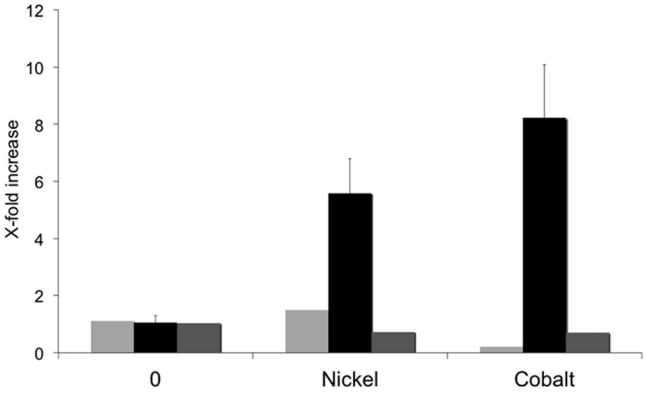

FIG 3 qRT-PCR analysis of expression of *R. leguminosarum dmeF* and flanking genes. Histograms correspond to qRT-PCR expression analysis of *orf03473* (light gray), *dmeF* (black), and *orf03476* (dark gray) genes in cells grown in standard TY culture medium (0) or in the same medium supplemented with nickel (200 μM $NiCl_2$) or cobalt (10 μM $CoCl_2$). Bars indicate standard errors from three experimental replicates.

Effect of *dmeRF* system on NiFe hydrogenase activity. Since it was previously shown that an Ni/Co metal efflux system, RcnRA, has an effect on NiFe hydrogenase in *E. coli*, the effect of inactivation of *dmeFR* genes on induction of hydrogenase activity in microaerobic free-living cells of *R. leguminosarum* was determined. In these assays, microaerobic cultures of the wild-type strain SPF25 induced normal levels of O_2-dependent H_2 uptake, whereas microaerobic cultures of D15 mutant exhibited significantly lower levels (ca. 50% reduction) (Table 4). This reduction in activity was not reverted by the addition of nickel at a concentration (1 μM) able to revert the low hydrogenase activity in mutant SPF22, devoid of both nickel transporter genes *hupE* and *hupE2* (47).

The effect of the deletion of the *dmeRF* system on the level of hydrogenase activity was also tested under symbiotic conditions. To this aim, the levels of hydrogenase activity in bacteroids from nodules induced in pea and lentil plants were determined (Table 5). In the case of pea, both wild-type and mutant strains induced normal levels of hydrogenase activity when plants were grown under standard nutrient conditions. Such levels were greatly enhanced by the addition of nickel, irrespective of the presence of the mutation in the *dmeRF* genes. The addition of cobalt resulted in

TABLE 3 Symbiotic expression of *R. leguminosarum dmeRF* genes

lacZ fusion	β-Galactosidase activity (Miller units)[a]					
	SPF25			D15		
	Control	Ni(II)	Co(II)	Control	Ni(II)	Co(II)
pDL43	33 ± 12	39 ± 11	33 ± 6	33 ± 5	43 ± 15	46 ± 13
pDL10	37 ± 12	35 ± 8	46 ± 7	40 ± 17	41 ± 13	113 ± 24
pDL13	56 ± 17	60 ± 17	63 ± 17	333 ± 114	367 ± 141	367 ± 74

[a] Values are β-galactosidase activities of pea bacteroids obtained from plants inoculated with *R. leguminosarum* SPF25 (wild type) and D15 (*dmeRF* deletion mutant) harboring *dmeF′-lacZ* (pDL43), *dmeRF′-lacZ* (pDL10), or *dmeR′-lacZ* (pDL13) fusions. Plants were grown in standard nutrient solutions (control) or in nutrient solutions supplemented with Ni(II) (85 μM $NiCl_2$) or Co(II) (42.5 μM $CoCl_2$). Values are the averages of four replicates ± standard errors.

partial inhibition (40% reduction) of hydrogenase activity in both wild-type and mutant strains. We also measured hydrogenase activity in bacteroids induced in lentil. In this host, the level of hydrogenase activity in plants grown under standard conditions was ca. 10 times lower than in pea, as we had previously observed (47). Again, the addition of nickel to lentil plants resulted in a 5-fold increase of hydrogenase activity, but interestingly, lentil bacteroids from the *dmeRF*-deficient strain induced significantly lower levels of hydrogenase activity under both standard and Ni-enriched conditions. The addition of cobalt resulted in a decrease of the activity, irrespective of the presence of the *dmeRF* deletion. These results suggest that bacteroids induced in lentil plants, but not in pea plants, require the *dmeRF* system to achieve an appropriate balance of intracellular nickel for expression of optimal levels of hydrogenase.

DISCUSSION

Active transport by efflux pumps is one of the most relevant mechanisms for metal resistance (48). Analysis of *R. leguminosarum* bv. viciae genome led to the identification of a *dmeF*-like gene. This gene encodes a member of the cation diffusion facilitator family. This family of metal-proton antiporters is involved in resistance to Zn(II) and other metals (12). *R. leguminosarum* DmeF presents a predicted topology of 6 TM domains, with two characteristic motifs (HX_3H at the beginning of TM2 and HX_3D at the beginning of TM5), and a histidine-rich stretch characteristic of the group of CDFs having Co(II) and Zn(II) as substrates (13) (see Fig. S1 in the supplemental material). Also, phylogenetic analysis placed the *R. leguminosarum* protein within the previously defined Zn-CDF group (13). Analysis of the *R. leguminosarum dmeRF*-deficient mutant indicates a major role for this protein in cobalt detoxification, whereas no effect on the tolerance to Zn(II) was observed in this mutant. This lack of effect on resistance to Zn(II) could be the result of the Co(II)- and Ni(II)-responsive regulation of *dmeF*.

TABLE 4 Effect of *dmeRF* genes on hydrogenase activity of *R. leguminosarum* bv. *viciae* SPF25

Strain	Relevant genotype	Hydrogenase activity with metal addition[a]	
		No metal	1 μM $NiCl_2$[a]
SPF25	Wild type	970 ± 170	1,000 ± 160
D15	SPF25 Δ*dmeRF*	510 ± 140	470 ± 190
SPF22	SPF25 Δ*hupE hupE2*	280 ± 50	1,060 ± 110

[a] Microaerobic cultures were assayed for hydrogenase activity after incubation under 1% oxygen for 16 h. Values are given in nmol of $H_2 \cdot h^{-1} \cdot$ mg of $protein^{-1}$ and represent the averages of four experiments ± standard errors.

However, assays carried out in the presence of cobalt levels leading to full induction of the system (10 μM) did not result in significant effects on resistance to zinc in a disk susceptibility assay (data not shown), suggesting either that *R. leguminosarum* DmeF has no relevant role in tolerance to zinc in this bacterium or that other systems providing resistance are present.

Analysis of data obtained by using *lacZ* fusions and by qRT-PCR determinations indicates that expression of *dmeRF* operon is strongly induced by nickel and cobalt in free-living cells. This is consistent with the presence of a gene (*dmeR*) encoding a protein homologous to RcnR, an *E. coli* nickel- and cobalt-responsive transcriptional regulator that, in the absence of nickel, represses synthesis of the efflux system RcnAB in this bacterium (17, 49). The unregulated high levels of expression of the system in the *dmeRF*-deficient mutant are corrected when the introduced fusion plasmid contains an active *dmeR* copy, suggesting that DmeR is actually a repressor whose effect is alleviated by the presence of these metals. Such a mode of regulation represents an alternative model to that described for *C. metallidurans*. In this bacterium, *dmeF* expression is constitutive and not inducible by metals (15). Conversely, the Ni and Co resistance *cnr* system described for the same organism shows an Ni-responsive regulation dependent on an alternative sigma factor (50).

It has been previously shown that nickel and cobalt binding to *E. coli* RcnR inhibits interaction of this protein with the *rcnAB* promoter region, thus removing transcriptional repression (45, 51). Sites critical for metal binding were mapped to residues His-3, Cys-35, His-60, and His-64 (45). All these residues are fully conserved in *R. leguminosarum* DmeR. However, the relative response to Ni(II)/Co(II) cations is different in *rcnR* versus *dmeR*. In the case of the *E. coli* system, RcnAB expression is induced to similar levels by Ni(II) and by Co(II) (45, 51), whereas in the case of *R. leguminosarum*, the level of induction of *dmeF* expression by Co(II) is higher than that by Ni(II). This difference might be due to the effect of sequence variations affecting residues other than those listed above. For instance, an E34Q mutation in RcnR yielded a higher response to cobalt (45). Also, recent evidence indicates that RcnR His-67 is involved in the interaction of RcnR with cobalt (20). These two residues are not conserved in the case of *R. leguminosarum dmeR*.

Our expression studies using *lacZ* fusions have shown that the relevant sequences for metal-induced expression of *dmeF* genes are located upstream of *dmeR*. Sequence analysis of this region in different *Rhizobium* species reveals the presence of a conserved palindromic sequence (ATA-X_2-ATA-C_6-TAT-X_2-TAT) (see Fig. S4 in the supplemental material). This sequence corresponds to the type I site (a single G/C tract flanked by an AT-rich palin-

TABLE 5 Effect of deletion or *R. leguminosarum dmeRF* gene on hydrogenase activity in symbiosis with different hosts

Strain	Relevant genotype	Hydrogenase activity in bacteroids from[a]:					
		Pea			Lentil		
		Control	Ni(II)	Co(II)	Control	Ni(II)	Co(II)
SPF25	Wild type	2,400 ± 140	6,650 ± 1080	1,470 ± 230	210 ± 50	1,190 ± 110	90 ± 10
D15	Δ*dmeRF*	2,870 ± 890	7,700 ± 890	1,020 ± 10	135 ± 20	580 ± 60	100 ± 10

[a] Values are given in nmol of $H_2 \cdot h^{-1} \cdot$ mg of protein^{-1}. Plants were grown with normal nutrient solutions (control) or with nutrient solutions supplemented with $NiCl_2$ (85 μM) or $CoCl_2$ (42.5 μM). Values are the averages of at least three replicates ± standard errors.

dromic sequence) proposed by Iwig and Chivers (19) for DNA binding of *E. coli* RcnR. Based on the constitutive expression associated with the absence of DmeR and on the existence of this potential binding site, a similar repression mechanism can be hypothesized for the control of *dmeF* expression in the absence of metals. In the case of endosymbiotic bacteria, genes for transcriptional repressor and efflux protein form a single operon, with the two genes transcribed in the same direction. In this case, the amount of repressor synthesized increases with the derepression of the system, thus allowing a tighter control of the regulation process than with the divergent promoter situation described to occur in *E. coli* for *rcnR-rcnA* genes. In that case, other modes of regulation might be also present that affect the expression of regulator and the regulated genes differently, as was exemplified by the differential regulation of *rcnR* and *rcnA* by iron (52).

Our results with *lacZ* fusions indicate a low level of expression of *dmeRF* genes in SPF25 pea bacteroids, even in the presence of added metals. This level of expression of the system was significantly induced in response to the presence of cobalt ions but only in the case of the *dmeRF* mutant. These data are consistent with the deleterious effect of high cobalt levels in the case of the mutant strain, suggesting the existence of a buildup of cytoplasmic metal concentrations in the absence of this efflux system. Our interpretation of these data is that the amount of metals actually available to the bacteroids is very low compared to the free-living situation. Even in this situation, a low level of expression of the DmeRF system is apparently required to maintain an adequate level of metals inside the bacteroids, since a significant decrease of plant growth was observed when pea plants inoculated with the *dmeRF*-deficient mutant were exposed to an excess of cobalt. The lower symbiotic performance of this mutant under high-metal conditions is consistent with a previous report on metal-susceptible mutants generated from *Bradyrhizobium japonicum* strains isolated from Ni-rich soils (53) and suggests that the mechanisms for metal resistance in the microsymbiont are relevant for the development of the symbiosis under high-metal conditions, at least in the case of pea. The situation is likely different in the case of lentil, where no impairment of plant symbiotic performance was associated with the deletion of the *dmeRF* system. This might reflect a lower exposure of lentil bacteroids to metals, as was concluded after analysis of the differential nickel-dependent limitations of NiFe hydrogenase in these two legume hosts (39).

The marked reduction in hydrogenase activity associated with the deletion of *dmeRF* system in free-living cells and lentil bacteroids is an unexpected result that might reflect an additional layer of complexity in the control of nickel homeostasis in this bacterium. Nickel is a key element for hydrogenase synthesis (43), and we had previously demonstrated that the deletion of nickel uptake transporter genes *hupE* and *hupE2* results in significant decreases in hydrogenase activity in free-living cells and in lentil bacteroids (47). A different situation was observed in the case of pea bacteroids. In this particular symbiosis there was no effect of HupE/HupE2 nickel transporters on the level of hydrogenase activity, suggesting the induction of a different mechanism for nickel provision in this symbiosis (47). Interestingly, we observe here a parallel pattern of results regarding the effect of the deletion of the *dmeRF* system on hydrogenase activity. It is tempting to speculate on the existence of an interaction between efflux and uptake systems, so both systems could be connected to maintain an optimal intracellular nickel level; such an interaction would not occur with the alternative uptake system proposed for pea bacteroids. The effect of a nickel efflux system in modulating the activity of other nickel enzymes, such as urease, has been previously documented for *Helicobacter pylori* (54), stressing the relevance of efflux systems for the maintenance of nickel homeostasis.

Analysis of the genome of other strains of *R. leguminosarum* bv. viciae, *Sinorhizobium meliloti*, *Rhizobium etli*, and *Agrobacterium tumefaciens* revealed that the metal efflux system presented in this work is likely conserved within the *Rhizobiaceae* (see Fig. S4 in the supplemental material). These data suggest that this model of a metal-inducible, RcnR-regulated CDF system has been selected by this group of bacteria as a general strategy for metal detoxification. Further studies are required to ascertain the actual role of this system in the maintenance of metal homeostasis and its relationship with metal availability for metalloenzyme biosynthesis in endosymbiotic bacteria.

ACKNOWLEDGMENTS

This work was supported by projects from Spain's MICINN (BIO2010-15301), Comunidad Autónoma de Madrid (S-505/AMB/0321 MICROAMBIENTE-CM), and Universidad Politécnica de Madrid [AL09-P(I+D)-06].

We thank Tomás Ruiz-Argüeso for critical reading of the manuscript.

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